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# Production and characterization of cellulolytic enzymes by *chaetomium globosum* for biomass saccharification and ethanol production

**Kelvin Masinde Munyasi**[1*], **George Isanda Omwenga**[1], **Fredrick Mjomba Mwamburi**[2]

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**Author Affiliation:**
[1]Biochemistry, Microbiology and Biotechnology Department Kenyatta University, P.O. Box 105285-00100, Nairobi, Kenya
[2]Pure and Applied Sciences Department Technical University of Mombasa, P.O Box 90420-80100, Mombasa, Kenya

[*]**Corresponding Author**
Biochemistry, Microbiology and Biotechnology Department Kenyatta University, P.O. Box 105285-00100. Nairobi, Kenya
Email: munyasi14@gmail.com

**Contact List**

| | |
|---|---|
| Kelvin Masinde Munyasi | munyasi14@gmail.com |
| George Isanda Omwenga | omwenga.george@ku.ac.ke |
| Fredrick Mjomba Mwamburi | mjmwamburi@yahoo.co.uk |

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## ABSTRACT

This study aimed to isolate, identify and characterize cellulase-producing fungi from a decaying tree trunk, and determine the effects of incubation time, moisture content and initial medium pH on cellulase production using untreated maize cobs and sugarcane bagasse under solid state fermentation after that, to saccharify for ethanol production. Samples were collected from Ngong Forest, Kenya, and screened for the isolation of cellulase producing fungi. The isolated fungi were grown to obtain pure cultures before DNA extraction, PCR amplification and sequencing. The fungus was cultured on substrates for cellulase production with enzymes obtained after fermentation subjected to cellulase assays; filter paper, exoglucanase and endoglucanase. Molecular data analysis was performed using the NCBI-BLAST algorithm and MEGA 11.0 software to identify the isolated fungus, while enzyme activity analysis was done using one-way ANOVA with R software at the P≤0.05 significance level, and the significant differences were determined via the Tukey post hoc test. The isolated fungus was identified as *Chaetomium globosum*. For the effect of incubation time on cellulase production, the fungus exhibited high enzyme production on different days within the incubation period, and the highest cellulase activity was recorded at a moisture content ratio of 1:2 and initial medium pH of 5. Saccharification optimization studies showed a 7% (v/v) enzyme concentration, 12% (v/w) substrate concentration and hydrolysis time of 72 hours were optimal for the maximum yield of reducing sugars. The total reducing sugar produced maximum bioethanol yield at 72 hours when *Saccharomyces cerevisiae* was used as a fermentation agent.

**Keywords:** Fungus, Cellulase, Enzymes, Production, Incubation, Moisture content, pH, *Chaetomium globosum*, Substrate, Reducing sugar, Fermentation, Ethanol

## 1. INTRODUCTION

Industrialization and a rapid increase in the global population over the past several decades have led to increased and exhaustive utilization of natural resources to meet the growing demand for energy consumption; as a result, there has been increased concern about environmental pollution and the exhaustion of fossil fuels (Bokinsky et al., 2011). Environmental compatibility is the most significant concern of nonconventional energy sources, as fossil fuel utilization hurts the environment due to the emissions of sulfur dioxide, carbon dioxide, and other poisonous gases (Ezeoha et al., 2017). To match the ever-increasing demand for energy, scientists have been compelled to find alternative renewable energy resources. Lignocellulosic plant biomass is an abundant, economical, and highly renewable natural resource available to humans and can be used to produce bioethanol, bioproducts, and heat.

Therefore, developing renewable energy from these resources as an option for fossil fuels will be necessary for the human population. The release of fermentable sugars from this lignocellulosic biomass is more challenging because it is costly and inefficient for producing lignocellulolytic enzymes. Cellulose is the main product of photosynthesis, and it is estimated that 100 billion dry tons of cellulosic biomass are produced annually (Srivastava et al., 2015). Plant biomass (plant dry weight) is composed of 35%- 50% cellulose, 5%-30% lignin and 20%-35% hemicellulose. Cellulose is a polysaccharide that contains glucose units that are bonded by β-1-4-glycosidic linkages Behera et al., (2017) and is made of repeated units of 15-45 glucans to make a fibril in a crystalline structure which combines to produce a macro-fibril that results in a form of alternate amorphous and crystalline regions. This structure increases the surface area and offers excellent resistance to degradation, which provides a hurdle for conversion to biofuels (Juturu and Wu, 2014).

The 1,4-glycosidic linkage of cellulose can be disrupted by cellulase enzymes, which have been used as efficient catalysts in most biotechnology processes as a substitute for inorganic catalysts (Khoshnevisana et al., 2017). To hydrolyze cellulose, cellulase enzymes are used to break down the insoluble cellulose macromolecules in plant biomass into simpler molecules of glucose, cellobiose, and cello-oligosaccharides, are required (Srivastava et al., 2015). Various microorganisms have been utilized for cellulase production (Srivastava et al., 2015). For example, *Trichoderma reesei* is considered the most efficient lignocellulose-degrading enzyme producers because they possess a developed intricate, and efficient degradation mechanism, comprising a large set of oxidative and hydrolytic enzymes that break down the complex structures of plant biomass (Benocci et al., 2017). Bioethanol production from plant biomass involves four stages: Pretreatment of the substrates, hydrolysis, fermentation of sugars, and recovery of products.

Hydrolysis of plant biomass is the most vital stage in biofuel production because it involves the conversion of complex carbohydrates to simple monomers. Hydrolysis can be performed using concentrated acids such as sulfuric acid or enzymes; however enzymatic hydrolysis is preferred since its cost-effective and generates higher yields (Akia et al., 2014). Cellulases are a set of enzymes that are produced by microorganisms such as bacteria and fungi and perform vital tasks in the carbon cycle by degrading insoluble cellulose to soluble sugars. These enzymes are essential in the food and drug industries, detergents, and textile production, paper and wood pulp industries and waste control and management (Jayasekara et al., 2019). Cellulase is composed of three different enzymes: endoglucanase, exoglucanase, and β-glucosidase. The cellulose hydrolysis process requires the three enzymes to work together synergistically (Zhang et al., 2006).

Endoglucanases hydrolyze the glycosidic bonds of cellulosic substrates at the same time, exoglucanase strikes the crystalline tails of cellulosic substrates to produce cellobiose, which is subsequently transformed into glucose molecules by the action of β-glucosidase. After hydrolysis, the reducing sugars obtained are fermented to manufacture different biofuels such as; bioethanol and biobutanol. Solid-state fermentation has become a significant discovery in the manufacture of microbial cellulase, and carbon substrates can be used as a solid matrix and potential carbon source for solid-state fermentation-based cellulase enzyme production. In this process, microorganisms are grown on a solid substratum in the absence or near absence of water; however, water is absorbed into the substrates to support microbial metabolism and growth.

It is designed to provide growth conditions that are equal to those of natural habitats, and thus, in comparison to other fermentation methods, it gives a greater productivity of enzymes. Solid-state fermentation is desirable and has many advantages, including low capital investment requirements, low technology or instrumentation requirements, easier purification of products, and high yielding. This study aimed at isolating, identifying and optimizing the production of cellulolytic enzymes from *Chaetomium globosum* using sugarcane bagasse, and maize cobs under solid-state fermentation for biomass saccharification and ethanol production.

## 2. MATERIALS AND METHODS

**Sample collection and preparation**

Decaying wood samples were collected from Ngong Forest, Nairobi (1°19'13"S, 36°44'54"E) in paper bags. The samples were transported to Kenyatta University Microbial Biotechnology Laboratory labeled appropriately, and stored at 4°C.

**Screening and isolation of cellulase-producing fungi**

The samples were divided into pieces (0.5 1 cm). Samples were sterilized for 30 seconds by 1% NaOCl, then rinsed five times using sterile distilled water and blot-dried using sterile paper towels. The samples were cultured on 1% Carboxymethylcellulose (CMC) media (Prepared from a mixture of 0.94 g CMC, 7.5 g Agar and 5 ml of 1% Congo red topped with sterile distilled water to 500 ml) in labeled Petri dishes, sealed using parafilm and incubated at room temperature. The plates were observed daily for fungal growth, emerging fungal cultures were subcultured on potato dextrose agar (PDA).

**Growing of isolated samples for pure culture**

Pure cultures of fungi were grown on potato dextrose agar (PDA). Two hundred grams (200 g) of cleaned, sliced potatoes were boiled for 30 minutes in 600 ml of distilled water. The broth was sieved using cheesecloth, and the volume was increased to 1000 ml with distilled water. Twenty grams of dextrose and fifteen grams of agar were added to the broth, and the mixture was autoclaved for 20 minutes at 121°C and 15 psi. After adding 50 mg/ml antibiotic (gentamycin), the PDA media was dispensed into disposable sterile Petri dishes in a lamina air hood. Pure cultures of cellulose-degrading fungi from CMC media dishes were subcultured on PDA media for use in DNA isolation and cellulose assays.

**Molecular identification of cellulolytic fungi**

*Genomic DNA extraction*

Total genomic DNA was extracted from pure cultures of fungi growing on PDA using the method described by (Lee et al., 2011). Fungal mycelia were removed using sterile scalpels, frozen, and subsequently ground into a fine powder with a mortar and pestle. The resultant powder was transferred into sterile 2 ml microcentrifuge tubes. A volume of 400 µL of lysis buffer (400 mM Tris- HCl [pH 8.0], 150 mM NaCl, and 1% sodium dodecyl sulfate) was added to the tube, which was subsequently stored at between 20 and 25°C for 10 minutes. The mixture was incubated in a water bath at 65°C for 15 minutes.

Equal amounts of chloroform: Isoamyl alcohol (24:1 v/v) were added, the contents were centrifuged at 13,200 rpm for five minutes at 4°C, and the supernatants were subsequently transferred to clean 2.0 ml microcentrifuge tubes. Equal amounts of isopropyl alcohol were added to the tube, and subsequently centrifuged at 13 200 rpm for 10 minutes; after that, the supernatant was removed. The DNA pellet was washed with 300 µl of 70% ethanol. The pellet was centrifuged at 10,000 rpm for 1 minute, after which the supernatant was removed. The DNA pellet was dried by aeration and dissolved in 50 µl of 1X Tris- EDTA (pH 8.0). The quality of the selected genomic DNA was estimated on 0.8% agarose and stored at -20°C until further use.

*PCR amplification*

PCR amplification of the 16S rDNA of internally transcribed fungal DNA regions was performed using the primers ITS 4 R (5'TCCTCCGCTTATTGATATGC-3') and ITS 86 F (5' GTGAATCATCGAATCTTTGAA-3') De-Beeck et al., (2014) in a PCR thermocycler. A total of 20 µl of PCR master mix was prepared comprising 12.2 µl of DNase- free H2O, 0.4 µl of forward primer, 0.4 µl of reverse primer, 4 µl of Taq, and buffer- My Taq polymerase (Bioline, USA), and 3 µl of DNA template. PCR condition was as follows for each cycle: 95°C for initial denaturation for 3 minutes, 95°C as denaturation temperature for 30 seconds, 50°C for annealing for 30 seconds, 72°C as extension temperature for one minute and, 72°C for final extension for seven minutes. A holding temperature of 4°C was used. PCR cycles were run for 35 cycles.

*Resolution of PCR products and sequencing*

The PCR products were separated using 1% agarose gel electrophoresis, then cleaned and sequenced using the Sanger dideoxy sequencing method by Microgen, Inc. (Netherlands).

**Inoculum preparation**

The selected pure fungal culture was grown on PDA media at room temperature. The cultures were incubated for seven days and used as inocula in the exponential growth stage. The fungal mycelia were scraped from the plates, mixed with sterile 5% glucose solution, and then blended using a sterile blender for 30 seconds. This mycelial mixture was utilized as a solid-state fermentation inoculum (SSF).

**Chemical analysis of the substrates**

Two substrates were used in solid-state fermentation; sugarcane bagasse and maize cobs. The substrates were broken down using a hammer mill and then sieved through a 2 mm sieve to obtain a standard uniform size. The chemical composition of the two untreated substrate samples was determined. The cellulose and lignin contents were analyzed by reaction with concentrated sulfuric acid based on a standard method recommended by TAPPI-T222 om-88. In contrast, the hemicellulose content was determined based on a standard way recommended by TAPPI T19m-54 standards (Motaung and Anandjiwala, 2015).

**Solid-state fermentation of untreated sugarcane bagasse and maize cobs**

Ten (10) ml Bushnell-Hass medium solution composed of 0.2 g MgSO4, 1.0 g Na2HPO4, 1.0 g KH2PO4, 1.0 NH4NO3, 0.1 g FeCl3.6H2O and 0.04 g CaCl2 was added to 1000 ml of distilled water, and the pH of the medium was adjusted to 5.0 using 1 M NaOH or 1 M HCL (Brijwani and Vadlani, 2011). The solution was subsequently added to 350 ml bottles containing five grams (5 g) of fine cellulosic substrates ground and sieved through a 2 mm sieve. The mixture's bottles were then sterilized at 121°C at 15 psi for 15 minutes (Brijwani and Vadlani, 2011). After cooling, the bottles were inoculated with 2 ml of the fungal inoculum 1x107 spores/ml. To determine the effects of the initial moisture content and pH on cellulase enzyme production, the moisturizing solution/wetting solution mixtures were prepared in the same way, but the moisturizing solution volume was varied ratios of 1:1, 1:2, 1:3, and 1:4. (w/v) and at pH 3, 5, 7, and 9 respectively.

**Extraction of Crude Enzymes**

The crude enzymes were harvested from solid -state fermentation by adding 50 ml of 50 mM citrate buffer (pH 4.8) to the flask. The flasks were shaken and left to stand at room temperature for two hours to extract the enzymes. Debris was filtered using cheesecloth, and the supernatant was centrifuged at 10,000 rpm for 10 min (Legodi et al., 2023). The supernatant was refrigerated at 20°C until use for enzyme bioassay activities. All the experiments were carried out in triplicate to ensure reproducibility.

**Effect of moisture content on cellulase production**

Initial substrate-to-moisture content ratio was investigated at 1:1, 1:2, 1:3, and 1:4 (w/v) at room temperature under static conditions. Five grams of untreated sugarcane bagasse and maize cobs were inoculated as indicated above (2.7). The fermentation progressed for an incubation period of 7 days under static conditions; after that, crude enzymes were extracted as described earlier (2.7).

**Effect of pH on cellulase production**

The effect of the initial medium pH was investigated in the range of 3, 5, 7, and 9 at room temperature under static conditions. Five grams of untreated sugarcane bagasse and maize cobs were inoculated as indicated above (2.8) for the fungal species. The fermentation progressed for an incubation period of 7 days under static conditions; after that, crude enzymes were extracted as described earlier (2.8).

**Cellulase activity assay**

***Filter Paper activity (FPase)***

One roll of Whatman No. 1 filter paper strip (1x 6 cm) was placed into a microcentrifuge tube, 500 µl of citrate buffer (pH 4.80) was added to the microcentrifuge tube, and 500 µl of the enzyme extract was added. The mixture was incubated at 50°C for 1 hour (Lee et al., 2011). After incubation, the reaction was stopped by adding 700 µl of 3,5-dinitrosalicylic acid reagent (3,5 DNS was prepared from a mixture of 3,5-dinitrosalicylic acid (10 g), sodium sulfite (0.5 g), and sodium hydroxide (10.0 g) adjusted to 1000 ml of distilled water); then, the mixture was boiled for five minutes in a water bath at 100°C. The tubes were then cooled to room temperature, and 300 µl of

potassium sodium tartrate was added. The reducing sugars produced during the reaction were determined spectrophotometrically at 540 nm against a reagent blank and estimated by a standard glucose curve. One unit of Fpase was defined as the volume of enzyme releasing one μmol of glucose sugar per minute.

***Endoglucanase assay***

Endoglucanase activity (CMCase) was determined by mixing 500 μl of 2% carboxymethylcellulose in 50 mM citrate buffer (pH 4.8) with 500 μl of filtered crude enzyme (Gautam et al., 2012). The reaction mixture was incubated at 50°C for 30 minutes in a water bath. The reaction was stopped by the adding 700 μl of 3,5-dinitrosalicylic acid reagent, and the mixture was subsequently boiled for precisely five minutes in a 100°C water bath. The tubes were left to cool at 21-25°C, after which 300 μl of potassium sodium tartrate was added. The reducing sugars produced were determined spectrophotometrically at 540 nm against a reagent blank and estimated by a standard glucose curve. One endoglucanase enzyme unit is expressed as the volume of enzyme generating one μmol of glucose per minute.

***Exoglucanase assay***

Exoglucanase activity was measured using a reaction mixture containing 500 μl of 1.25% w/v Avicel in 100 mM sodium acetate buffer (pH 4.8) and 500 μl of enzyme extract. (Gautam et al., 2012). The reaction mixture was incubated at 50°C for two hours in a water bath. The reaction was stopped by adding 700 μl of 3,5- DNS reagent, and the reaction mixture was boiled for precisely five minutes in a 100°C water bath. The tubes were cooled to room temperature, after which 300 μl of potassium sodium tartrate was added. The reducing sugars produced were determined spectrophotometrically at 540 nm against a reagent blank and estimated by a standard glucose curve. One unit of endoglucanase activity was defined as the volume of enzyme releasing one μmol of glucose sugar per minute.

**Saccharification for reducing sugar determination**

The crude enzyme extract was used to hydrolyze sugarcane bagasse and maize cobs; the crude enzyme was concentrated 5-fold by freeze drying. Saccharification was carried out using 50 ml Falcon tubes in a 20 ml total volume containing 7% cellulase enzyme and 1%, 5%, 10% and 15%, w/v sugarcane bagasse, maize cobs and rice husks. The tube contents were agitated at 150 rpm, and samples were collected at 0, 12, 24, 36, 48, 60 and 72 hours to determine the amount of reducing sugars produced. The amount of reducing sugars produced was determined by the 3,5-DNS reagent (Zhang et al., 2009). The absorbance was measured spectrophotometrically at 540 nm, and the amount of reducing sugars produced was quantified using a glucose standard curve.

**Ethanol production from sugarcane bagasse and maize cobs**

Ethanol was produced from saccharified sugarcane bagasse and maize cobs by Separate hydrolysis (SHF) and fermentation in 50 ml Falcon tubes in a 20 ml total volume using *Saccharomyces cerevisiae.* Samples were taken at 12, 24, 36, 48, 60, and 72 hours, and the amount of ethanol produced was quantified using potassium dichromate. The potassium dichromate reagent solution was prepared by adding 125 ml of distilled water to a 250 ml volumetric flask and then carefully adding 70 ml of concentrated sulfuric acid while constantly swirling the flask.

Then, 0.75 grams of potassium dichromate was added to the solution, and subsequently topped with distilled water to 250 ml. The alcohol content was calculated by the potassium dichromate method. The general principle of this method is that potassium dichromate is a yellowish solution that reacts with alcohol in the presence of sulfuric acid to form a green complex due to the reduction in potassium dichromate, which is thus directly proportional to the alcohol content. The absorbance was measured at 600 nm Williams et al., (1950) using a Jenway 6300 spectrophotometer, an ethanol standard curve was used to quantify the amount of ethanol produced.

**Data Management and Analysis**

All the experiments were carried out in triplicate. The spectrophotometer readings were converted into international units (IU) using a glucose standard curve, entered into Excel spreadsheets, imported into R-software version 4.0.5 and evaluated for compliance with parametric assumptions. The data obtained were analyzed by one-way ANOVA at the $P<0.05$ significance level. If a significant difference in the factors was noted, Student–Newman–Keuls post hoc tests were used to determine the sample groups that differed significantly. In molecular data analysis, lines were matched to sequences in the nucleotide database (NCBI) by the BLAST algorithm.

Neighbor-joining phylogenetic analysis was performed with the MEGA software version 11.0 and R version 4.4.1 to determine the relationships among the fungal species (Kumar et al., 2018). The data are presented in tables and graphs.

## 3. RESULTS

**Molecular identification of cellulolytic fungi**

To conduct molecular identification, DNA was extracted from the fungal isolates. PCR of 28S rDNA was conducted using the primers ITS 4 R (5′TCCTCCGCTTATTGATATGC-3′) and ITS 86 F (5′ GTGAATCATCGAATCTTTGAA-3'), and the PCR products were subsequently sent to Macrogen, Inc., for NGS. Table 1 shows the details of its identity. The samples were analyzed via BLAST, and further phylogenetic conclusions were drawn with the help of MEGA X (Kumar et al., 2018). The phylogenetic tree is shown (Figure 1).

**Table 1** The fungal isolate was identified as *Chaetomium globosum.*

| Genebank Accession no. | Description | Identity | Information |
|---|---|---|---|
| MH645800.1 | *Chaetomium globosum* | 99% | C.*globosum* belongs to the *Chaetomium* fungal genus which contains approximately 80 known species, family *Chaetomiaceae*, and division Ascomycota. They are filamentous fungi mainly found on rocks, air, soil, decaying plant materials, and herbivore dung. Although it is soil borne, it also commonly infest water-damaged buildings. |

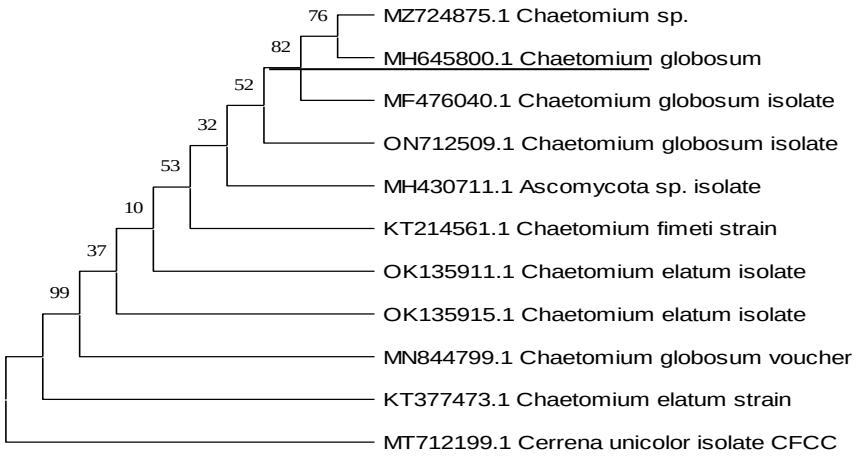


**Figure 1** The phylogenetic tree of *Chaetomium globosum.*

Phylogenetic analysis of the 18S rDNA sequence of *Chaetomium globosum* sequences of related strains was obtained from NCBI-BLAST. A phylogenetic tree was created using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa were clustered together according to the bootstrap test (1000 replicates) is shown above the branches. The evolutionary distances were computed using the Kimura 2-parameter method Tamura et al., (2021) and are expressed in units of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA11 (Kumar et al., 2018).

**Substrate composition**

The substrate compositions used for cellulose enzyme production and ethanol fermentation were collected from a single batch and analyzed. The results are reported as the dry weight of four significant components; cellulose, hemicellulose, lignin and extractives (Table 2). Among the two substrates, sugarcane bagasse had the highest cellulose and hemicellulose contents at P>0.05 (49.3±1.8% and 31.2±0.7%, respectively), while maize cobs had the highest lignin (18.1±1.2%).

**Table 2** Percentage composition of substrates

| Substrates | Treatments | Cellulose (%w/w) | Hemicellulose (%w/w) | Lignin (%w/w) | Extractives (%w/w) |
|---|---|---|---|---|---|
| Maize cobs | Untreated | 48.1±1.6 | 26.2±0.4 | 18.1±1.2 | 7.9±0.16 |
| Sugarcane bagasse | Untreated | 49.3±1.8 | 31.2±0.7 | 12.6±0.5 | 6.8±0.06 |

Note: The data are expressed as the mean ± SEM of triplicate sample measurements.

**Effect of incubation time on the cellulase enzyme production of *Chaetomium globosum* cultured on sugarcane bagasse and maize cobs**

Cellulase-degrading fungi were studied to determine the effect of incubation time on endoglucanase activity (CMcase), exoglucanase activity (Avicel), and filter paper activity (FPase) by culturing them on sugarcane bagasse and maize cobs as carbon substrates at room temperature for different intervals (3, 6, 9, 12 and 15 days). The highest cellulase FPase activity was 17.23±0.21 IU/ml on the 3rd day on sugarcane bagasse, significantly more than the FPase activity recorded on the remaining days of incubation. For the maize cobs, the highest FPase activity was recorded as 11.31±0.39 IU/ml on the 6th day of incubation, which was not significantly greater than that recorded on the 3rd day (Table 3).

The highest endoglucanase activity was 49.42±0.49 IU/ml, recorded on the 3rd day for sugarcane bagasse, which was not significantly greater than that recorded on the 6th day of incubation. For the maize cobs, the highest endoglucanase activity was 37.56±2.62 IU/ml, recorded on the 6th day of incubation; this value was significantly greater than that of the remaining days of incubation (Table 4). The highest exoglucanase activity produced was 10.25±0.56 IU/ml observed on the 3rd day on sugarcane bagasse, which was not significantly greater than that recorded on the 6th day but was considerably greater than that recorded on the 9th and 12th days of incubation. For the maize cobs, the highest exoglucanase activity was 5.78±0.30 IU/ml, which was recorded on the 6th day of incubation and was not significantly different from that recorded on the 3rd day of incubation (Table 5).

**Table 3** Effect of incubation time on the FPase activity of *Chaetomium globosum* cultured on sugarcane bagasse and maize cob substrates.

| Fungus | Substrate | Day 3 | Day 6 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|---|
| *C. globosum* | SB | 17.23±0.21aA | 16.38±0.16aB | 11.63±0.19aC | 10.99±0.15aC | 2.78±0.11bD |
| | MC | 10.58±0.30bA | 11.31±0.39bA | 7.07±0.30bB | 7.65±0.36bB | 5.34±0.12aC |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row are significantly different at P≤0.05 for each substrate.

**Table 4** Effect of incubation period on Endoglucanase activity of *Chaetomium globosum* cultured on sugarcane bagasse and maize cobs substrates.

| Fungus | Substrate | Day 3 | Day 6 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|---|
| *C. globosum* | SB | 49.42±0.09aA | 45.68±0.06aA | 24.89±0.13aC | 30.14±0.13aB | 3.89±0.03bD |
| | MC | 23.84±0.04bB | 37.56±0.02bA | 13.77±0.17bCD | 15.90±0.20bC | 10.69±0.09aD |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Standards expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Means expressed with different superscript capital letters within the same row are significantly different at P≤0.05 for each substrate.

**Table 5** Effect of incubation time on exoglucanase activity *Chaetomium globosum* cultured on sugarcane bagasse and maize cob.

| Fungus | Substrate | Day 3 | Day 6 | Day 9 | Day 12 | Day 15 |
|---|---|---|---|---|---|---|
| *C. globosum* | SB | 10.25±0.56aA | 9.68±0.31aA | 5.96±0.12aB | 5.75±0.18aB | 1.73±0.16bC |
| | MC | 5.75±0.09cA | 5.78±0.30bA | 3.57±0.08bB | 3.93±0.07bB | 3.21±0.6aB |

Note: SB= Sugarcane bagasse and MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Standards expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Means expressed with different superscript capital letters within the same row are significantly different at P≤0.05 for each substrate.

**Effect of moisture content on cellulase production by *Chaetomium globosum* cultured on sugarcane bagasse and maize cobs.**

Cellulase-producing fungi were studied to determine the effect of substrate moisture content on exoglucanase activity, filter paper activity, and endoglucanase activity by culturing them on sugarcane bagasse, and maize cobs as carbon substrates. To determine the effect of moisture content; the substrates were moistened with Bushnel Haas media at different solid-to-liquid ratios ranging from 1:1 to 1:4. The highest FPase activity was 8.56±0.49 IU/ml, which was recorded at a substrate: moisture ratio of 1:2 for sugarcane bagasse; this value was not significantly greater than that recorded at a substrate: Moisture ratio of 1:3 but was considerably greater than that of the other remaining levels. For the maize cobs and sugarcane bagasse, the highest FPase activities were 5.75±0.29 IU/ml and 4.42±0.34 IU/ml, respectively, which were recorded at a substrate: moisture ratio of 1:2 and were significantly greater than those recorded for the other remaining ratios (Table 6).

The highest endoglucanase activity was 11.8±0.45 IU/ml with a substrate: moisture ratio of 1:2 for sugarcane bagasse, which did not differ significantly from that with a ratio of 1:3. For the maize cobs, the highest endoglucanase activity was 8.24±0.27 IU/ml, recorded with a substrate: Moisture ratio of 1:2, which was comparable to that recorded with a substrate: Moisture ratio of 1:3 (Table 7). The highest exoglucanase production was 9.36±0.71 IU/ml after seven days of incubation at a moisture content ratio of 1:2 on sugarcane bagasse, which was not significantly greater than that at a moisture content ratio of 1:3 but was substantially greater than that recorded at moisture content levels of 1:1 and 1:4. For the maize cobs, the highest exoglucanase activity was 6.21±0.33 IU/ml at a moisture content of 1:2, which was significantly greater than that recorded at moisture contents of 1:1, 1:3 and 1:4 (Table 8).

**Table 6** Effect of the substrate: moisture ratio on the total cellulase activity (FPase) of *Chaetomium globosum* cultured on sugarcane bagasse, and maize cobs.

| Substrate: Moisture ratio | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 1:1 | 1:2 | 1:3 | 1:4 |
| *Chaetomium* | SB | 4.58±0.33aB | 8.56±0.49aA | 7.35±0.39aA | 5.59±0.22aB |
| *globosum* | MC | 1.19±0.14bC | 5.75±0.29bA | 3.28±0.16bB | 2.77±0.23bB |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ± SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Table 7** Effect of the substrate: moisture ratio on the endoglucanase activity of Chaetomium globosum cultured on sugarcane bagasse, and maize cobs.

| Substrate: Moisture ratio | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 1:1 | 1:2 | 1:3 | 1:4 |
| *Chaetomium* | SB | 6.96±0.23aC | 11.8±0.45aA | 11.4±0.23aA | 10.0±0.22aB |
| *globosum* | MC | 2.15±0.34bC | 8.24±0.27bA | 7.34±0.16bA | 4.06±0.08bB |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscript small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Table 8** Effect of the substrate: moisture ratio on the exoglucanase activity of *Chaetomium globosum* cultured on sugarcane bagasse, and maize cobs.

| Substrate: Moisture ratio | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 1:1 | 1:2 | 1:3 | 1:4 |
| *Chaetomium* | SB | 5.21±0.29aC | 9.36±0.71aA | 8.13±0.38aAB | 5.74±0.66aBC |
| *globosum* | MC | 1.89±0.24bC | 6.21±0.33bA | 4.18±0.28bB | 2.99±0.28bBC |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscript small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Effect of pH on cellulase production by *Chaetomium globosum* cultured on sugarcane bagasse and maize cobs.**

Cellulase-degrading fungi were studied to determine the effect of the initial pH of the substrate on endoglucanase activity, exoglucanase activity and filter paper activity by culturing the fungus on sugarcane bagasse and maize cobs as carbon substrates at four pH, values 3, 5, 7 and 9 of the moisturizing solution. The highest cellulase FPase activity was 8.72±0.18 IU/ml at pH 3 for sugarcane bagasse, which was not significantly different from that recorded at pH 7 and 9. For the maize cobs, the highest filter paper activity was 5.55±0.39 IU/ml at pH 5, significantly different from that recorded at the other pH levels (Table 9).

The highest endoglucanase activity was recorded at pH 5.0 (12.20±0.13 and 8.67±0.60) for all the substrates, sugarcane bagasse, and maize cobs, respectively, which were significantly greater than those at the other pH values (Table 10). The highest exoglucanase activity after seven days of incubation was 10.8±0.55 IU/ml, recorded at pH 9 on sugarcane bagasse; this activity was not significantly greater than that at pH 5, but was considerably greater than that at the other pH values. For the maize cobs, the highest exoglucanase activity was 5.41±0.35 IU/ml, recorded at pH 5, which was significantly different from that recorded at the other concentrations (Table 11).

**Table 9** Total cellulase (FPase) activity of Chaetomium globosum cultured on sugarcane bagasse and maize cobs.

| pH | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 3 | 5 | 7 | 9 |
| *Chaetomium* | SB | 8.72±0.18aA | 5.78±0.31aB | 8.35±0.55aA | 8.20±0.25aA |
| *globosum* | MC | 1.31±0.23bBC | 5.55±0.39aA | 1.70±0.11bB | 0.58±0.09bC |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Table 10** Effect of endoglucanase from *Chaetomium globosum* cultured on sugarcane bagasse, and maize cobs.

| pH | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 3 | 5 | 7 | 9 |
| *Chaetomium* | SB | 9.15±0.36aB | 12.20±0.13aA | 6.85±0.28aC | 9.15±0.54aB |
| *globosum* | MC | 2.10±0.19bBC | 8.67±0.60bA | 3.07±0.38bB | 1.10±0.04bC |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column are significantly different at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Table 11** Effect of exoglucanase activity of *Chaetomium globosum* cultured on sugarcane bagasse, and maize cobs.

| pH | | | | | |
|---|---|---|---|---|---|
| Fungus | Substrate | 3 | 5 | 7 | 9 |
| *Chaetomium* | SB | 8.57±0.66aAB | 10.0±0.56aA | 6.25±0.47aB | 10.8±0.55aA |

| | | | | | |
|---|---|---|---|---|---|
| *globosum* | MC | 1.51±0.12bB | 5.41±0.35bA | 1.80±0.03bB | 1.61±0.18bB |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Effect of substrate loading on reducing sugar production**

The effects of varying concentrations of substrates on reducing sugar production were studied with other variables held constant. There was an increase in the amount of reducing sugars produced from 12 hours to 72 hours during saccharification of maize cobs, and sugarcane bagasse using 5 FPU of *C. globosum* crude enzyme extract. On saccharification of maize cobs using 50 FPU of *C. globosum* cellulase, significantly more reducing sugars were produced after 72 hours of saccharification. The highest reducing sugars for 1%, 5%, 12% and 15% substrate loading were 24.85±1.08 g/L, 37.01±1.10 g/L, 39.20±0.81 g/L and 35.81±0.92 g/L, respectively, at 72 hours of saccharification, which were significantly greater than those produced between 12 and 60 hours of saccharification.

Overall, the highest reducing sugars were made at a substrate load concentration of 12% when maize cobs were used as substrates (Table 12). For reducing sugars produced on sugarcane bagasse, more significant amounts were made at 72 hours after saccharification at 1%, 5%, 12%, and 15% substrate loading represented by 25.41±0.05 g/L, 37.92±1.11 g/L, 51.17±0.68 g/L and 46.96±0.37 g/L, respectively. Overall, the highest reducing sugars were produced at a substrate load concentration of 12% when sugarcane bagasse was used as a substrate (Table 12).

**Table 12** Shown are the different substrate concentrations of the enzymes produced by *Chaetomium globosum*.

| Substrate | Load conc. | 12 hours | 24 hours | 36 hours | 48 hours | 60 hours | 72 hours |
|---|---|---|---|---|---|---|---|
| Maize cobs | 1% | 13.81±0.43cE | 15.81±0.04cDE | 17.83±0.27cD | 20.18±0.11cBC | 22.16±0.10cB | 24.85±1.08bA |
| | 5% | 17.54±0.50bE | 18.72±0.13 bE | 22.66±0.35bD | 27.17±0.08bC | 31.44±0.89bB | 37.01±1.10aA |
| | 12% | 21.81±0.17aC | 23.83±0.55aC | 26.5±0.20aC | 34.53±0.06aB | 36.20±0.37aB | 39.20±0.81aA |
| | 15% | 10.48±0.47dE | 19.17±0.49bD | 21.12±0.51bD | 29.12±0.09bC | 32.88±0.31bB | 35.81±0.92aA |
| Sugarcane bagasse | 1% | 10.74±0.07cD | 14.77±0.09bcCD | 17.38±0.19dC | 19.62±0.70cAC | 21.09±0.70cB | 25.41±0.05cA |
| | 5% | 15.68±0.12bC | 18.34±0.09bC | 19.60±0.18cC | 21.57±0.76cC | 30.18±0.07bB | 37.92±1.11bA |
| | 12% | 23.67±0.09aD | 25.76±0.08aCD | 27.41±0.11aC | 29.36±0.58aC | 42.82±0.07aB | 51.17±0.68aA |
| | 15% | 21.14±0.12aE | 22.32±0.09aDE | 23.92±0.12bD | 25.12±0.21bC | 33.78±0.02bB | 46.96±0.37aA |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row are significantly differ at P≤0.05 for each substrate.

**Effect of enzyme loading on reducing sugar production**

The effects of varying concentrations of enzymes, on reducing sugar production were studied while the other variables were held constant. The results of different enzyme concentrations (0%, 1%, 2%, 5%, and 7%) on reducing sugar production were also variable for cellulases from *C. globosum*. There was an increase in the amount of reducing sugars produced from 12 hours to 72 hours during the saccharification of maize cobs, and sugarcane bagasse using different enzyme loads of 0%, 1%, 2%, 5% and 7% v/v of *C. globosum*. The production of reducing sugars increased steadily with time and enzyme concentration.

The highest amounts of reducing sugars from the enzymes produced by *C. globosum* on maize cobs and sugarcane bagasse for all five different enzyme load concentrations (0%, 1%, 2%, 5%, and 7% v/v) were 3.14±0.46 g/L, 32.0±0.35 g/L, 28.6±0.17 g/L, 36.6± 0.35 g/L, and 38.3±0.39 g/L, respectively, for maize cobs; and 4.05±0.24 g/L, 47.0±0.49 g/L, 45.5±0.23 g/L, 45.7±0.81 g/L, and 47.9±0.58 g/L, respectively, for sugarcane bagasse, all of which were recorded after 72 hours and were not significantly greater than those recorded at 60 hours. Overall, reducing sugars were produced at the highest level at an enzyme load concentration of 7% at all recorded times compared to the other enzyme load concentrations (Table 13).

**Table 13** Effects of different enzyme concentrations produced by *Chaetomium globosum* on sugarcane bagasse, and maize cobs.

| Substrate | Load conc. | 12 hours | 24 hours | 36 hours | 48 hours | 60 hours | 72 hours |
|---|---|---|---|---|---|---|---|
| Maize cobs | 0% | 2.72±0.34cA | 3.09±0.37dA | 3.41±0.35cA | 3.25±0.18dA | 3.09±0.12dA | 3.14±0.46dA |
| | 1% | 17.7±0.56bD | 21.5±0.44cC | 30.7±0.39bB | 34.7±0.68bA | 32.6±0.45bAB | 32.0±0.35bB |
| | 2% | 19.6±0.37bD | 29.5±0.55aBC | 30.8±0.45bAB | 31.4±0.32cA | 28.3±0.24cC | 28.6±0.17cC |
| | 5% | 24.1±0.39aD | 27.1±0.09bC | 35.9±0.19aB | 37.0±0.56aA | 36.1±1.18aAB | 36.6±0.35bA |
| | 7% | 21.4±1.75abC | 26.7±0.47Bb | 37.0± 0.49Aa | 38.0±0.56Aa | 38.7±0.35Aa | 38.3±0.39Aa |
| Sugarcane bagasse | 0% | 4.37±0.51dA | 4.18±0.42dA | 4.02±0.35dA | 3.33±0.65cA | 3.60±0.19bA | 4.05±0.24cA |
| | 1% | 23.8±0.51cD | 28.2±0.57cC | 31.6±0.77cB | 46.1±0.41bA | 46.2±0.28aA | 47.0±0.49aA |
| | 2% | 25.9±0.48bcD | 29.5±0.44bcC | 41.4±1.33bBC | 46.3±0.49bA | 44.8±1.04aAB | 45.5±0.23bAB |
| | 5% | 27.0±0.63bD | 31.3±0.62bC | 34.1±0.77cC | 47.0±0.39aA | 45.5±0.93aB | 45.7±0.81bB |
| | 7% | 29.8±0.28aD | 45.4±0.67aB | 47.6±0.16Aab | 48.8±0.47aA | 47.6±0.46aAB | 47.9±0.58aA |

Note: SB= Sugarcane bagasse, MC=Maize cobs. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Separate hydrolysis (SHF) of reducing sugars for ethanol production**

Optimum (maximum) substrate concentration and enzyme load were used to saccharifize maize cobs and sugarcane bagasse. The highest amount of reducing sugar produced from fermentation and saccharification of enzymes produced by *C. globosum* and yeast on sugarcane bagasse was 4.23±0.12 g/L, recorded on the 12th hour, which was not significantly different from the highest value recorded from that of commercial enzymes (4.45±0.17 g/L) at the same hour. For the maize cobs, the highest reducing sugar concentration was 3.19±0.26 g/L at 12 hours, significantly lower than the reducing sugar concentration produced by commercial enzymes (4.51±0.22 g/L) (Table 14).

**Table 14** Shows the results of amount of reducing sugars during separate hydrolysis and fermentation of maize cobs, and sugarcane bagasse.

| Substrate | 12 hours | 24 hours | 36 hours | 48 hours | 60 hours | 72 hours |
|---|---|---|---|---|---|---|
| SB (a) | 4.23±0.12aA | 3.98±0.06aAB | 3.30±0.43bB | 3.12±0.06aB | 1.98±0.02bAC | 1.46±0.06bC |
| (c) | 4.45±0.17aA | 4.17±0.07aA | 4.56±0.24aA | 3.36±0.06aB | 2.30±0.03aAC | 1.98±0.03aC |
| MC (a) | 3.19±0.26bA | 2.67±0.05bAB | 2.47±0.16bBC | 2.30±0.04aBC | 1.94±0.08aCD | 1.42±0.09bD |
| (c) | 4.51±0.22aA | 3.45± 0.05aB | 2.98±0.39aBC | 2.46±0.05aCD | 2.25± 0.05aCD | 1.94± 0.06aAD |

Note: SB= Sugarcane bagasse, MC=Maize cobs. a=crude enzymes and c=commercial enzymes. The values are presented as the means of 3 replicates ±SEMs and are expressed as IU/ml. Means expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

**Ethanol production from sequential fermentation**

Ethanol production was achieved by fermentation of reducing sugars and yeast over 72 hours. The highest amounts of ethanol produced from fermentation and saccharification of enzymes produced by *C. globosum* fermented with yeast on sugarcane bagasse and maize cobs were 8.78±0.08 g/L and 9.56±0.08 g/L, respectively, at 72nd hour. These amounts were not significantly different from those produced by commercial enzymes or yeast on sugarcane bagasse, and maize cobs which were 8.93±0.27 g/L and 9.97± 0.09 g/L, respectively, at the same time (Table 15).

**Table 15** Results of ethanol production by S. cerevisiae using separate hydrolysis fermentation by hydrosylates of sugarcane bagasse and maize cobs.

| Substrate | 12 hours | 24 hours | 36 hours | 48 hours | 60 hours | 72 hours |
|---|---|---|---|---|---|---|
| SB (a) | 6.56±0.2aC | 6.54±0.1aC | 6.68±0.2aC | 7.54±0.11aB | 7.92±0.1bAB | 8.78±0.08aA |
| (c) | 7.03±0.13aC | 7.03±0.12aC | 7.34±0.06aC | 7.70±0.29aBC | 8.41±0.09aAB | 8.93±0.27aA |
| MC (a) | 6.65±0.33aC | 6.86±0.1bBC | 6.94±0.34aBC | 7.04±0.13bBC | 7.79±0.09aB | 9.56±0.08aA |
| (c) | 7.62±0.18aC | 7.83± 0.03aC | 8.23±0.09aBC | 8.24±0.12aBC | 8.52± 0.21aB | 9.97± 0.09aA |

Note: SB= Sugarcane bagasse, MC=Maize cobs. a= crude enzymes and c=commercial enzymes. The values are presented as the means of 3 replicates ±SEMs and are expressed as g/L. The means are expressed with different superscripted small letters in the same column significantly differ at P≤0.05 for each fungus. Standards expressed with different superscript capital letters within the same row significantly differ at P≤0.05 for each substrate.

## 4. DISCUSSION

Lignocellulosic plant biomass comprises complex biological materials that consist of agricultural refuse (sugarcane bagasse, wheat/rice straw, rice husk, maize cob), forestry refuse, and office refuse (Kumar et al., 2009). These renewable resources are available in unlimited supply, with an annual estimated collection of approximately 200 billion metric tons, of which only 3% are fully exploited, and often in nonfood production, such as the pulp and paper industries (Zhang et al., 2009; Fukuda et al., 2009). Since these materials are not utilized in the human food chain, lignocellulosic plant biomass is a relatively low-cost feedstock and a perfect source of reducing sugars for dependable bioethanol production through the development of lignocellulose-based biorefineries (Rezania et al., 2019).

Lignocellulose is composed primarily of plant cell wall materials that consist of cellulose (10-25%), hemicellulose (20-35%) and lignin (35-50%), depending on the biomass of the plant (Zoghlami and Paës, 2019). Cellulases are categorized into three main groups based on their mode of action and biochemical structure. They are: Endoglucanases, exoglucanases, and β-glucosidases which act synergistically on cellulose in lignocellulosic biomass to convert it into glucose. Cellulose degradation by cellulase is affected by different factors, such as the type of enzyme, quantity of enzymes, quality of substrate, and environmental factors (temperature, pH, and nutrients) (Hong et al., 2007). Cellulases have a broad range of uses in various industries, such as; the paper and pulp industry, the food industry, and the production of bioethanol via the conversion of cellulose to fermentable sugars.

The process of biomass conversion relies on the utilization of efficient enzymes for the degradation of celluloses into simple sugars that are easily fermented into biofuels due to the vast and abundant distribution of sugarcane bagasse, maize cobs, and rice husks not only in Kenya but also worldwide as agricultural refuse; additionally, it is vital to utilize these enzymes as substrates for efficient conversion of locally isolated fungi to produce fungal cellulases. Taking into account the advantages of the process of solid-state fermentation, we investigated several of the parameters that affect cellulase production during the degradation of sugarcane bagasse, and maize cobs such as; (i) the fermentation incubation period, (ii) the medium moisture content, (iii) the initial pH and (iv) the comparison of lignocellulosic substrates. Moreover, we investigated the optimum substrate concentrations and enzyme concentration for reducing sugar production and subsequent ethanol production.

**Molecular identification of cellulolytic fungi**

The ITS rDNA region sequence is an essential tool for identifying fungal species isolated from environmental sources and it thus has been widely used for detecting fungal communities and improving classical identification (Anderson and Parkin, 2007). In this study, ten different fungal strains were screened for the cellulase enzyme production. The fungus possessing the highest cellulolytic ability, was identified both morphologically and on a molecular basis. Molecular identification was carried out by DNA barcoding using ITS region sequencing. The ITS rDNA sequences were subsequently compared to those in the databases using NCBI-BLAST, and PCR of 28S rDNA was conducted using ITS 4(R) and ITS 86 (F). BLAST analysis indicated that 99% of the isolates belonged to *Chaetomium* sp. and was 99% similar to that of *Chaetomium globosum* (GenBank accession number: MH645800.1) (Boratyn et al., 2019).

**Composition of the untreated substrates**

The substrate compositions used for cellulase enzyme production and ethanol fermentation were collected from a single batch and analyzed; the results are reported as the dry weight of three crucial components; cellulose, hemicellulose, lignin and extractives. Cellulose, hemicellulose and lignin are important constituents of substrates because they are responsible for yielding fermentable sugars upon hydrolysis by carbohydrates. The maize cob composition consisted of 48.1±1.6% cellulose, 26.2±0.4% hemicellulose, and 18.1±1.2% lignin; these results contrast with those of a previous study by Gandam et al., (2022), whose reported that the composition of maize cobs was 33–43% cellulose, 26–36% hemicellulose, and 17–21% lignin.

The sugarcane bagasse comprised of 49.3±1.8% cellulose, 31.2±0.7% hemicellulose, and 12.6±0.5% lignin. These results are in agree with those of a study by Mahmud and Anannya, (2021), who reported sugarcane bagasse contents of approximately 40–50% cellulose, 25–35% hemicellulose, and 9–25% lignin. Previous studies have shown differences in the compositions of substrates due to some factors, such as: the geographic conditions from which the substrates were obtained, the different parts of the substrates used, various growth states of the substrates and the different methods used for drying of the substrates (Gandam et al., 2022). These results demonstrated that maize cobs and sugarcane bagasse could be good carbohydrate sources.

**Effect of Time Course on Enzyme Production**

The optimal incubation time for enzyme production depends on the type of substrate used and the composition of the moisturizing medium, the initial pH of the medium, and the different fungal species utilized for enzyme production (Ilyas et al., 2011; Milala et al., 2005). The best cellulase production results recorded from this study, were obtained when untreated sugarcane bagasse was used in solid-state fermentation compared to maize cobs. The optimum incubation time for cellulase production varied for different isolates even though the media used were the same (Soeka and Ilyas, 2020). Enzyme production depends on biomass, but only during the exponential fungal growth phase is maximum production achieved. As cellulases are primary metabolites, they are produced at high yield during the exponential phase of growth, and at the onset of the death phase, enzyme secretion starts decreasing (Dutt and Kumar, 2014).

Total cellulase (FPase) is used to quantify the general cellulose hydrolyzing capacity of cellulase mixtures (Delabona et al., 2013). FPase activity is conveyed as a filter paper unit, with one unit of enzymatic activity being expressed as the volume of enzyme producing one μmol of reducing sugar per minute. *C. globosum* cultured on sugarcane bagasse and maize cobs as substrates exhibited the highest FPase activity after three days; after that, the activity decreased consistently with increasing incubation time. This is attributed to the increase in the production of byproducts resulting from microbial metabolism and nutrient depletion, which inhibited the growth of the fungi and the formation of the enzyme after that (Shafique et al., 2009). These results are in agreement with a study by Santos et al., (2016) using *Aspergillus niger* and *Rhizopus* sp, who recorded the highest FPase activity after three days when grown on prickly pear as a substrate under solid-state fermentation.

Similarly, Chakraborty et al., (2016) investigated the production of cellulose hydrolyzing enzymes by *Trichoderma* sp. RCK65 under SSF culture on a *Prosopis juliflora* substrate achieving the highest FPase production on the 3rd day of incubation. *C.globosum* on the sugarcane bagasse and maize cob substrates all exhibited the highest endoglucanase activity after three days. Thereafter, the action did not increase with an increasing incubation time, possibly due to the depletion of nutrients or the production of toxic substances that prevent the growth of organisms and subsequent endoglucanase production (Abdullah et al., 2021). These results are in agreement with those of a study by Santos et al., (2016), who investigated *Aspergillus niger* and *Rhizopus* sp. grown on prickly pear and rice husk under solid-state fermentation and their effect on endoglucanase activity for 5 days; the authors obtained the highest endoglucanase activity after 3 days of incubation.

Similarly, Chakraborty et al., (2016) recorded the highest level of endoglucanase production on the 3rd day when the *Trichoderma* sp. strain was cultured under SSF conditions using a *Prosopis juliflora* substrate. The optimum incubation time for endoglucanase activity varied for the different isolates cultured in the same medium, possibly due to the depletion of different macro- and micronutrients in the fermentation medium throughout the incubation time, which strained the physiology of the fungi and caused inactivation of the endoglucanase enzyme secretion machinery. Exoglucanase functions in a continuously on the reducing and non-reducing tails of the cellulose chain, releasing soluble cellobiose. *C. globosum* grown on the sugarcane bagasse and maize cob substrates exhibited the highest exoglucanase activity on the 3rd day of incubation.

Similar findings were reported in a study by Sherief et al., (2010), who investigated the influence of incubation time on exoglucanase production by *Emericella niveus* cultured on rice straw and wheat bran under SSF and recorded peak exoglucanase activities on the 3rd day; another study by who investigated the effect of incubation time on exoglucanase production by *Trichoderma harzianum* cultured on sugarcane bagasse in natura and obtained the highest exoglucanase activity on the 3rd day of incubation. The general reduction in cellulase production after the optimum maximum temperature is reached could be due to the following reasons: a decrease in moisture content over time, denaturation of the enzyme as a result of changes in pH during fermentation, or the accumulative effect of cellobiose, which inhibits enzyme production (Melo et al., 2007; Singh et al., 2009).

The optimal incubation period also depends on the type and composition of the medium used in the SSF, the initial pH, and the different fungal species utilized for cellulase production (Milala et al., 2005; Ilyas et al., 2011). These findings further suggested that the growth and quantity of cellulase enzymes after fermentation were markedly different for the different lignocelluloses. However, compared with the maize cobs, the maximum amounts of CMCase, exoglucanase (FPase), and endoglucanase were detected in the sugarcane bagasse. These differences may be attributed to the nutrient accessibility and chemical nature of the substrates (Sherief et al., 2010).

**Effect of pH on cellulase production**

All enzymes have an optimal pH at which they produce the maximum activity, after which their activity decreases with increasing or decreasing pH. It was observed that the production of cellulase enzymes gradually increased with increasing pH until it reached the maximum or optimum pH and then reduced (Ye et al., 2017). Among the various physical parameters, the pH of the moisturizing media plays an essential role in inducing morphological changes in hyphal growth and mycelia in fungi and in enzyme secretion (Sethi and Gupta, 2014). The pH variation observed during the growth of microorganisms also impacts enzyme stability in the medium. The optimal pH varies with different microorganisms and enzymes. The isolate was allowed to grow in media with various pH values ranging from 3 to 9.

*C. globosum* exhibited maximum FPase activity at pH 7 for sugarcane bagasse, which was in agreement with the findings of a previous study by Yakubu and Vyas, (2021) on the influence of medium pH by adjusting the pH of the moisturizing medium used to wet the substrate. An initial medium pH 7 produced the maximum FPase activity on substrates for *Mucor circinelloides*. For maize cobs as substrate, the highest FPase activities were recorded at pH 5, which was in agreement with the results of a previous study by Sethi and Gupta, (2014), where the influence of pH on FPase production by *Aspergillus niger* cultured on agricultural wastes was also recorded at pH 5, and another study by Beldman et al., (1985), where *T. viride* produced the highest FPase at pH 5 when cultured on plant biomass. The endoglucanases produced exhibited maximum activity at pH 5, with a further increase in pH reduced the endoglucanase activity.

The decrease in endoglucanase activity at higher pH was probably due to proteolytic inactivation of cellulase. Hence, slightly acidic pH values favored cellulase production by the fungus, which further increased the pH, and the cellulase activity decreased gradually, which is in agreement with earlier results of other researchers (Dutt and Kumar, 2014). In this study, *C. globosum* grown on sugarcane bagasse and maize cobs exhibited the highest endoglucanase activity at pH 5, which is in agreement with the results of a study by Jalal et al., (2014) in which *Scytalidium thermophilum* achieved the highest endoglucanase activity on agro wastes at pH 5-6, with no significant difference between them and another study by Moretti et al., (2012), who investigated the ability of thermophilic fungi to produce cellulases and recorded the highest endoglucanase activity by *Myceliophthora thermophila* on plant biomass at an optimum pH of 5. *C. globosum* exhibited the highest exoglucanase activity at pH 5 for maize cobs and sugarcane bagasse, after which it decreased; this decrease in exoglucanase activity may be due to variation or imbalance in the proton motive force of the membrane.

Which was in agreement with the findings of studies performed by Mukherjee et al., (2011), who reported that exoglucanase activity from *Rhizopus oryzae* cultured on sweet lime peel was most excellent at pH five and was stable over a pH range of 5–9. Olajuyigbe, (2017) studied the effect of pH on exoglucanase production and reported that the highest activity occurred at pH 5 for *Fusarium oxysporum*. These observations also agree with the findings of Li et al., (2013), who studied the effect of exoglucanase production from *Trichoderma reesei* on corn cob and reported the highest production at pH 5. The optimum pH of the medium is an essential parameter for microbial growth and enzyme production. A pH value lower or higher than the optimum pH will affect the metabolic activities of the microorganism. It also affects the stability of the enzyme and can cause protein denaturation (Kalra and Sandhu, 1986). From this study, pH of 5 was optimal and was therefore used for the remainder of the study.

**Effect of moisture content levels**

The moisture content during solid-state fermentation is an essential factor that enables successful enzyme production. The moisture content at which free moisture occurs varies considerably among different substrates concerning their water-binding qualities. The impact of moisture content on microbial growth and cellulase formation may be attributed to the effect of moisture on the physical characteristics of the solid substrates (Shah and Madamwar, 2005). A moisture content higher than the optimum may cause a reduction in porosity, alteration of the particle structure, a gummy texture, decreased oxygen transfer, and enhancement of aerial mycelia (Gervais et al., 2003). The moisture level in the fermentation medium defines microbial growth and enzyme biosynthesis. Water enables microorganisms to uptake nutrients from solid substrates. Additionally, the effectiveness of mass transfer in solid-phase particles is dependent on moisture and substrate qualities; however, an excessive increase in moisture content decreases enzyme production since an increase in water past the optimum leads to depletion of the contact surface of substrate particles.

In addition, the water film condenses, causing a deficiency in the air supply to the substrate particles (Raimbault and Alazard, 1980). The solubility of nutrients in solid substrates, water absorption, and swelling of substrates are reduced when the moisture content is lower than the optimum value, which results in reduced enzyme production (Dutt and Kumar, 2014). *C. globosum* strain cultured on sugarcane bagasse and maize cobs exhibited the highest FPase activity at a substrate: moisture content of 1:2 after incubation, during which the moisture level decreased or increased beyond or below a 1:2 ratio, decreasing the FPase activity. This reduction in FPase activity could be due to a lower moisture content causing a reduction in the solubility of nutrients on the different substrates, a low degree of swelling, and a greater water tension on the substrates. Higher moisture content leads to a reduction in FPase activity, due to steric hindrance of fungal growth by reducing the inter-particle spaces of the solid matrix, hence inhibiting the rate of oxygen transfer (Mrudula and Murugammal, 2011).

This finding is in agreement with that of a study reported by Mrudula and Murugammal, (2011), in which the highest FPase activity occurred at a moisture content level of 1:2 for *Aspergillus niger* under solid-state fermentation on coir waste as a substrate. The effect of moisture content levels on endoglucanase production was influenced by the fungal species. In this study, *C. globosum* exhibited the highest endoglucanase production at a moisture content of 1:2 after incubating with the substrates, after which the activity decreased considerably, which could be attributed to the decrease in the rate of oxygen transfer with increasing moisture content. Similar results were obtained by Mrudula and Murugammal, (2011), who reported the highest endoglucanase activity at a moisture content of 1:2 for *A. niger* cultured on coir waste under solid-state fermentation.

*C. globosum* grown on sugarcane bagasse and maize cobs exhibited the highest exoglucanase activity at a substrate: moisture ratio of 1:2. A further increase in moisture level above or lower than a 1:2 ratio resulted in a decrease in exoglucanase activity. This more significant increase in production at lower moisture may be due to preferential synthesis of specific proteins, such as proteins from cellulase enzymes, at lower moisture levels than at higher moisture levels and a decrease in oxygen transfer at higher moisture contents. Similar observations were also reported by Subhosh and Reddy, (2013), who recorded the highest exoglucanase activity at a moisture content of 1:2 in response to *A. niger* growth on wheat bran.

**Effect of enzyme concentration on reducing sugar production**

The cost of cellulases is proportional to the total cost of the saccharification process. Thus, it is recommended to utilize the minimal enzyme dosage in the saccharification process (Chen et al., 2008). To investigate the effect of cellulase loading on reducing sugar production in untreated maize cobs and sugarcane bagasse, the impact of different cellulase concentrations (1%, 2%, 5%, and 7% (v/v)) on the enzymatic hydrolysis of fixed quantities (5 gms) of the untreated substrates were investigated. The impact of reducing sugar production was studied over 72 hours. An increase in enzyme loading appeared to favor the enzymatic hydrolysis of maize cobs and sugarcane bagasse substrates. The highest amounts of reducing sugars recorded from this study were obtained with cellulases from *C. globosum* at 7% (v/v) concentration from all three substrates (Maize cobs and sugarcane bagasse) via solid-state fermentation. The effect of enzyme concentration on reducing sugar production was influenced by the fungal species.

In this study, cellulases produced by *C. globosum* grown on sugarcane bagasse and maize cobs had the highest reducing sugar production at 60 hours of incubation and a 7% enzyme concentration, indicating an increase in the production of reducing sugars with an increase in incubation, resulting in no significant increase in sugar production. This may be due to limited substrate availability for enzyme attack, thus decreasing the conversion efficiency (Mohd et al., 2019). These results are in agreement with those of a study by Abdullah et al., (2021), who optimized conditions for fermentation of sugarcane bagasse with cellulase enzymes produced from

*Aspergillus niger* under solid-state fermentation conditions for the enzymatic hydrolysis of sugarcane bagasse and reported a rise in reducing sugar yield with increasing enzyme concentration. As such, a cellulase loading of 7% was considered the optimum value and was adopted throughout the rest of the experiments.

**Effect of substrate concentration on reducing sugar production**

The reducing sugar production from each of the substrates increased significantly based on the substrate concentration used in enzymatic hydrolysis up to a substrate concentration of 12% (w/v), after which no significant increase was recorded, irrespective of the substrate used. This effect is because high substrate concentration results in lower reducing sugar yields due to the inhibitory effect of the byproducts/end products released in high quantities (Xin et al., 2013). The highest reducing sugars were detected in this study when sugarcane bagasse was used solid-state fermentation after 72 hours of incubation and at a 12% substrate load concentration for enzymes produced by *C. globosum*, implying an increase in reducing sugars with increasing substrate concentration and incubation time, these findings show that the low concentration is due to minimal hydrolyzation of biomass.

This observation was similar to that of a study by where reducing sugars were produced from the fermentation of sugarcane bagasse and sawdust with cellulases produced from White Rot Fungi, and more reducing sugars were obtained from a larger volume of substrates. Similarly, a study by Idrees et al., (2014) obtained the same results, in which fermentable sugars were produced by combined chemoenzymatic hydrolysis of plant biomass to reduce sugar production and subsequent ethanol production. For maize cobs as a substrate, the highest reducing sugars were detected after 72 hours of incubation, and at a 12% substrate load, the enzymes produced by *C. globosum* exhibited a general increase in hydrolysis with increasing substrate concentration and incubation time; as the substrate load increased, more substrate was available for enzymatic conversion until an optimum was achieved; thereafter, any further increase in the concentration of the substrates past 12% (w/v) did not result in any addition in reducing sugar production.

This could be because of mass transfer effects or end-product inhibition (Geng et al., 2012). These results were in agreement with those of a study by Fanyin–Martin et al., (2022), who investigated the various parameters of optimal production of reducing sugars by hydrolysis by commercial enzymes using mango peels and papaya and pineapples as substrates and achieved increased reducing sugar production at the highest substrate concentration tested. A further study by Xin et al., (2013) produced reducing sugars from the fermentation of horticultural wastes (vegetable refuse, tree trunks, and branches) with cellulase enzymes produced from T. *reesei* and it revealed that further increases in the concentrations of the substrates increased the amount of reducing sugars produced. A 12% (w/v) substrate that resulted in the highest production of reducing sugars, was selected as the substrate concentration for ethanol production in subsequent studies.

**Ethanol production using sequential saccharification and fermentation.**

The fermentability of the saccharification products was further evaluated by using *S. cerevisiae* as the fermenting organism over 72 hours. *S. cerevisiae* was chosen because it is a very efficient ethanolic fermenter, capable of fermenting glucose from the breakdown of cellulose (Liu et al., 2010). The yield of ethanol increased with increasing fermentation time as the ethanol concentration increased, as the sugars decreased because *S. cerevisiae* consumed the sugar and converted it into alcohol and CO2. The more available the sugars were, the higher the ethanol production yield was. From the results, I observed that bioethanol production increased steadily during all the monitored hours to achieve the highest peak in the 72nd hour, and this was the general trend observed across all the substrates, indicating that the sugars released during the saccharification; were readily converted to ethanol. This observation is similar to that of who studied impact of different *S. cerevisiae* concentrations on ethanol production from oil palm fronds and reported an increase in ethanol production with fermentation time and a proportional decrease in reducing sugars.

In another study by Abada et al., (2018), bioethanol was produced with cellulases from *Bacillus cereus* isolated from sesame seed residue, with *S. cerevisiae* being used for fermentation, and an increase in the production of ethanol was recorded with increasing fermentation time. Additionally, similar findings were achieved by studied the sequential saccharification and fermentation of corn stover for biofuel production using the wood-rot fungus *Escherichia coli* K011 and *Saccharomyces cerevisiae* and recorded a steady increase in ethanol production with fermentation time, with the highest ethanol yield being recorded at 96 hours. A comparison of the effectiveness of fungal-produced crude enzymes to that of commercial enzymes in ethanol production revealed that the average yield of *C. globosum* was not significantly greater at the optimum time points recorded than of commercial enzymes when the same unit was used for activity.

These results are in agreement with the findings of a study conducted by Xin et al., (2013), who used crude enzymes produced from solid-state fermentation of the fungus *T. reesei* RUT-C30 and horticultural wastes (tree trunks and branches, wheat straws, and vegetable refuse) as substrates. The increase in production following the use of crude enzymes might be because the crude enzyme solutions possessed greater cellulase activities than the commercial enzymes, which could contribute to the greater reducing sugar concentration (Xin et al., 2013). These results proof that the crude enzymes from the fungi have a bioethanol production potential, comparable to that of commercial enzymes; and hence can be used to produce reducing sugars for ethanol production.

## 5. CONCLUSION

From this study, the following conclusions can be drawn;

*C. globosum* isolated from decaying tree trunks is a cellulase enzyme-producing fungus.

An incubation time of 3 days is optimal for cellulase enzyme production from *C. globosum* using untreated sugarcane bagasse and maize cobs.

pH and moisture content had different effects on cellulase enzyme production by *C. globosum*.

Cellulase enzymes produced by *C. globosum* can saccharify sugarcane bagasse and maize cobs into simple sugars.

Saccharified sugarcane bagasse and maize cob can be fermented into ethanol.

**Abbreviations**

ANOVA: Analysis of variance

BLAST: Basic Local Alignment Search Tool

CMC: Carboxymethylcellulose

DNA: Deoxyribonucleic acid

DNS: 3,5-dinitrosalicylic acid

EDTA: Ethylenediaminetetra acetic acid

KALRO: Kenya agriculture and livestock research organization

NCBI: National Center for Biotechnology Information

PCR: Polymerase chain reaction

PnPG- P-nitrohenyl-β-D-glucopyranosidase

SSCF: Simultaneous saccharification and co-fermentation

SSF: Solid state fermentation

SSSF: Saccharification and simultaneous fermentation

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**Author Contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kelvin Masinde Munyasi and Dr. George Isanda Omwenga. The first draft of the manuscript was written by Kelvin Masinde Munyasi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Ethical approval**

This study does not contain human or animal subjects performed by any of the authors.

**Informed consent**
Not applicable.

**Conflicts of interests**
The authors declare that there are no conflicts of interests.

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**Data and materials availability**
All data associated with this study are present in the paper.

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